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SINGLE CHAIN ANTIBODY (SCA) ENCODING GENES: ONE-STEP CONSTRUCTION AND EXPRESSION IN EUKARYOTIC CELLS

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We report the expression, in eukaryotic cells, of a gene encoding a single chain antibody (SCA) and a rapid method for the construction of such genes. A SCA directed against the aromatic dye fluorescein was synthesized from a gene constructed by means of the simultaneous use of four PCR primers and templates of both light and heavy chain immunoglobulin cDNAs in the form of either plasmid clones or reverse transcribed hybridoma RNA. Two of the primers were partially complementary to one another and encoded the polypeptide linker which joins the immunoglobulin light and heavy chain variable domains of the SCA polypeptide. A functional, hapten-binding product was synthesized from the gene thus constructed in both *E. coli* and the fission yeast, *Schizosaccharomyces pombe*. Our results demonstrate that gene constructs encoding single chain antigen binding proteins can be synthesized very rapidly with only limited sequence information about the pertinent light and heavy chain immunoglobulin genes, and, that neither murine codon usage bias, *Thermus aquaticus* DNA polymerase infidelity, nor the eukaryotic cellular environment preclude the synthesis of functional single chain antigen binding proteins in non-lymphatic, non-murine eukaryotic cells.

Single chain antigen binding proteins (or single chain antibodies, SCAs) consist of immunoglobulin light and heavy chain variable domains tethered by a polypeptide linker[1,2]. Because SCAs can bind cognate antigens or haptens with affinities approaching those of their parent monoclonal antibodies, SCAs may have many applications where effector functions mediated by immunoglobulin constant regions are unnecessary or are a liability. For example, SCAs directed against a cell type-specific surface antigen have been coupled to an immunotoxin, thereby targeting the toxin to a unique cell type[3].

We are interested in exploring the physiological consequences of expressing single chain antibodies in transgenic, non-lymphatic eukaryotic cells. To this end, we have developed a polymerase chain reaction (PCR)-based method for rapidly synthesizing SCA-encoding genes and have evaluated expression of such constructs in an easily manipulated and rapidly assayed eukaryote, the fission yeast, *Schizosaccharomyces pombe*. Our gene synthesis method is similar to "splicing by overlap extension"[4] but involves the use of PCR not only to fuse light and heavy chain immunoglobulin gene sequences but also to incorporate a new polypeptide encoding domain between them. The process is greatly simplified by the findings that both templates and all primers can be mixed in a single reaction mixture and that reverse transcribed hybridoma RNA can be used as template. We also show that such constructs can be expressed in eukaryotic cells to yield functional, antigen-binding proteins.

RESULTS

The fluorescein hapten system. Monoclonal antibody 4-4-20 exhibits high affinity for the polycyclic aromatic hapten fluorescein. The relative affinity has been determined to be 1.8×10^{10} M^{-1} for this antibody-antigen complex[5]. Binding of 4-4-20 to fluorescein results in a marked (up to 95%) reduction in the latter's fluorescence at 535 nm when excited by an actinic wavelength of 493 nm. Complementary DNA clones encoding the 4-4-20 variable light and heavy chains have been obtained and fully sequenced[6]. In addition, the crystal structure of the 4-4-20 monoclonal antibody has recently been reported[7]. Just as some of these features made the 4-4-20/fluorescein system an attractive model for the design and synthesis of the first single chain antigen binding protein (SCA)[1], we chose this system for further development of SCA gene synthesis technology and *in vivo* eukaryotic expression studies.

One-step construction of 4-4-20 SCA-encoding gene. In order to facilitate the manipulation and analysis of alternative SCA designs, we developed a PCR-based method for the construction of SCA-encoding genes that avoids their assembly from oligonucleotides as reported in previous studies[1,2]. Based on the published sequence of the 4-4-20 variable regions and that of the SCA linker peptide previously reported[1,6], we designed sets of primers for the PCR-based synthesis of a 4-4-20 SCA-encoding gene, incorporating features for its subsequent manipulation and expression in *E. coli*. We chose to synthesize a gene which would encode a SCA with the structure V_L-linker V_H. (The construction of SCAs of the opposite structure, V_H-linker-V_L, has also been reported[2]). Two pairs of oligonucleotide PCR primers were made, one pair to prime V_L synthesis and one for V_H synthesis (see Experimental Protocol). Each primer consisted of 20 nucleotides of perfect 3' complementarity with its respective template, based on published 4-4-20 F_V sequences[6]. The 5' "tails" of the V_L antisense (VLANTI) and V_H sense (VHSENSE) primers consisted of 45 nucleotides perfectly complementary to one another. These 45 bases encode the 15 amino acid peptide which joins the V_L and V_H domains of the SCA. VLANTI and VHSENSE incorpo-

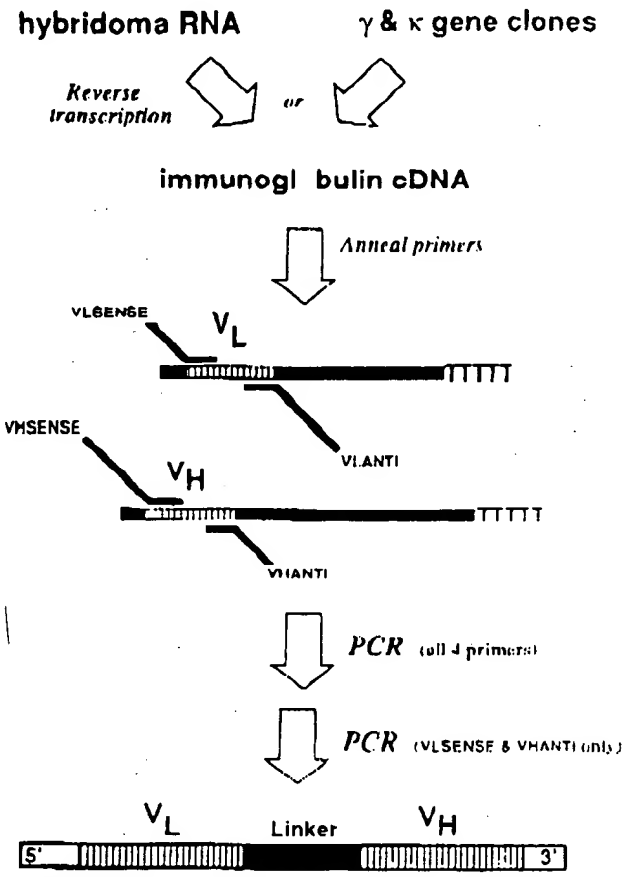

FIGURE 1 Scheme for PCR-based synthesis of single chain antibody encoding genes. Entire procedure is carried out in a single tube through the first PCR synthesis. One tenth of the first PCR product is supplied as templates for the second PCR synthesis. See text for details.

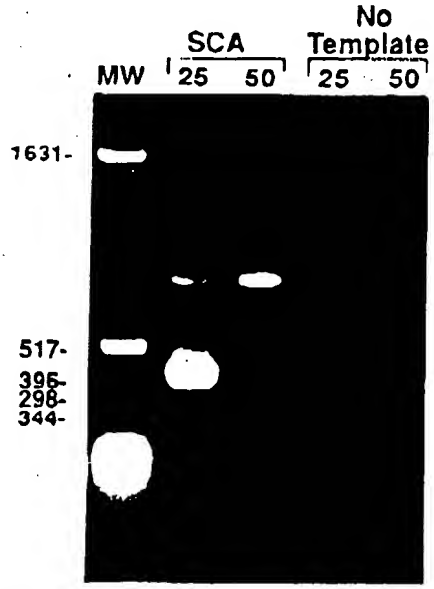

FIGURE 2 Products of SCA gene synthesis from plasmid kappa and gamma clone templates. 1.5% agarose gel in TEA buffer was stained with ethidium bromide. Lanes contain (left to right): Std. plasmid pBR322 digested with *HinfI*; SCA (25 & 50), PCR products of 4-4-20 IgG cDNA plasmid clone templates, after 25 and 50 cycles of PCR; No Template (25 and 50), same as previous, except template omitted.

rated *XhoI* and *HindIII* sites, respectively, at the linker-F_v junctions, to facilitate future linker swapping experiments. The 5' "tail" of the V_L sense primer (VLSENSE1) carried stop codons in all three reading frames and an optimally situated consensus ribosome binding site[8]. The V_L sense (VLSENSE1) and V_H antisense (VHANTI) primers carried 5' distal *BamHI* and *EcoRI* sites, respectively, as well as terminal "spacer" segments to facilitate the cloning of PCR products.

The scheme for PCR construction of the 4-4-20 SCA-encoding gene is outlined in Figure 1. Plasmid pUC19 derivatives carrying cDNA copies of the 4-4-20 IgG light and heavy chain encoding genes were combined with all four oligonucleotide primers in a single PCR synthesis. Following 25 cycles of PCR, a 10 μl aliquot of crude PCR product was removed and added to a second PCR reaction mixture containing only the V_L sense (VLSENSE1) and V_H antisense (VHANTI) primers. This second PCR synthesis consisted of an additional 25 cycles with a PCR profile identical to that of the previous synthesis (see Experimental Protocol). The products of the first and second 25 cycles of PCR are shown in Figure 2. The predominant products of the first 25 cycles are the individual V_L and V_H domain-encoding segments (appox 350 bp each). A minor product seen at ca. 750 bp is the full-length SCA encoding gene. A subsequent set of 25 PCR cycles led to substantial enrichment for full-length product and greatly facilitated its cloning (Fig. 2). The ca. 750 bp product was gel-purified and ligated into pUC119 yielding plasmid pWD1.

It is now possible to determine the sequences of IgG-encoding genes without cloning them[9, 10]. Therefore, we explored SCA gene synthesis directly from reverse-transcribed hybridoma mRNA. We performed a PCR synthesis identical to that described above, but with a template of reverse-transcribed RNA isolated from 4-4-20 hybridoma cells. The products of this synthesis are shown in Figure 5. The products of these PCR syntheses are essentially identical to those obtained with cloned IgG genes as templates. Total hybridoma cellular RNA appears to provide an adequate reverse transcription template.

Verification of the PCR-synthesized SCA-encoding gene. The scheme for PCR-based SCA synthesis was validated by structural and functional analysis of the cloned gene's product, and, ultimately, by DNA sequence analysis. The 4-4-20 SCA-encoding gene was expressed, under *lac* control, in *E. coli*. Protein products were visualized by probing immunoblots with polyclonal anti-4-4-20 antisera (Fig. 4). An ca. 27 kD product, detected by such a reagent, was present in the lysate of *E. coli* carrying pWD1, but absent from that carrying the vector pUC119. This protein product was functionally tested by assessing its ability to bind fluorescein-conjugated Sepharose. Figure 4 shows that an *E. coli*(pWD1) lysate contains an ca. 27 kD product, which can be selectively removed by incubation with fluorescein-Sepharose. Therefore, the PCR-synthesized gene encodes a functional antigen-binding protein. Finally, the entire DNA sequence of the PCR product cloned in pWD1 was determined and was found to conform perfectly with the predicted sequence (data not shown).

Expression of SCA gene product in fission yeast. The SCA structural gene in pWD1 was modified by PCR to generate an insert which was cloned into the fission yeast translational fusion vector pEACE2, yielding pBD1 (see Experimental Protocol). Expression of the 4-4-20 SCA from pBD1 in *S. pombe* provided a test of SCA synthesis in a non-lymphatic eukaryotic cell. Using procedures identical to those employed to detect functional SCA gene products in *E. coli* lysates, above, a fluorescein-binding

protein was sought in transgenic fission yeast. The immunoblot in Figure 5 shows that a ca. 27 kD protein can be selectively removed from lysates of transgenic *S. pombe* with fluorescein-Sepharose and that this protein is detectable with anti-4-4-20 antiserum. Therefore, the functional, fluorescein-binding SCA protein is also expressed in *S. pombe*.

DISCUSSION

Clinical and diagnostic applications, as well as the prospects for catalytic antibodies[11], continue to promote interest in monoclonal antibodies. Many of these applications require neither the constant domains nor the tetrameric structure of the IgG molecule. Single chain antibodies (SCAs) have therefore found favor in the molecular immunology arena. In this paper, we have demonstrated a facile procedure for the construction of genes encoding SCA proteins, and have shown that functional products can be recovered when such genes are expressed in *E. coli* and fission yeast.

While this work was in progress, a similar PCR-based gene fusion method was reported[1] which differs from our method in two aspects. First, we have shown that a yield of full-length products sufficient for cloning is obtained by combining all four primers and freshly synthesized cDNA in a single PCR synthesis. Second, we incorporated a linker peptide-encoding segment into the "inside" primers. Construction of a SCA-encoding gene by a relatively more tedious PCR-based approach has also been reported[3].

The product of reverse transcription of 4-4-20 hybridoma RNA provides a suitable pair of light and heavy chain cDNA templates for SCA gene construction (Fig. 3). This is a powerful option for PCR-based SCA gene synthesis, since the sequences of immunoglobulin mRNAs can be determined directly from hybridoma mRNA[9, 10], obviating the need to clone the respective mRNAs as cDNAs. The abundance of immunoglobulin mRNA in hybridoma message pools suggests that template limitation will not preclude faithful sequence reproduction by *Thermus aquaticus* DNA polymerase.

It is customary and sufficient for PCR primers to have less complementarity with their templates (e.g., 20 nucleotides) than the full 15 nucleotides of our "inside" primers' 5′ tails. Were the mutual complementarity of our two "inside" primers confined to their 5′ terminal 21 nucleotides, then these primers could have each been made 12 bases shorter, or primers of the same length could have encoded a linker 8 amino acids longer.

In a previous report, a 4-4-20 SCA identical to that synthesized here was recovered from inclusion bodies in *E. coli*[1]. That we obtained functional SCA proteins from French press lysate supernatants does not imply that our SCA products from *E. coli* were more soluble than those previously described. Our experiments simply demanded far less protein than the kinetic studies reported[1]. Further study will be required to quantify the synthesis levels and site(s) of intracellular localization of the 4-4-20 SCA gene products in fission yeast cells. We have recently observed that incorporation of an alternate, longer linker peptide significantly enhances SCA production in *E. coli* (G.T.D., unpublished results).

Degenerate oligonucleotide primers[12], mixed primers complementary to relatively conserved regions upstream and downstream from the IgG variable domain sequences[13,14], and "universal" primers complementary to these same regions[15], can be used for cloning of immunoglobulin cDNAs by PCR. Application of such techniques in conjunction with the synthesis scheme reported

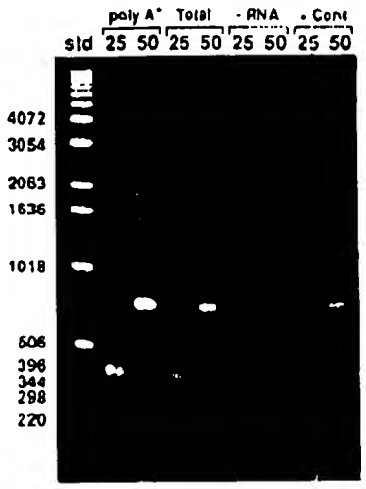

FIGURE 3 Products of SCA gene synthesis by PCR using reverse transcribed hybridoma cell RNA templates. Gel conditions as in Figure 2. 25 and 50 refer to 25 and 50 cycles of PCR, respectively. Lanes contain (left to right): Std, BRL 1 kb ladder; poly A+, reverse transcribed poly A+ hybridoma mRNA as template; Total, total unfractionated hybridoma RNA as template; No RNA, no RNA added to reverse transcriptase reaction; + Cont, positive control PCR.

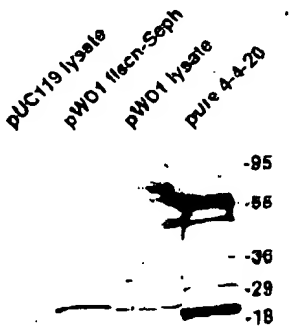

FIGURE 4 Immunoblot of 4-4-20 SCA gene products expressed in *E. coli*. Signals visualized by alkaline phosphatase coupled goat anti-rabbit IgGs and appropriate chromogenic substrates. Lanes contain (left to right): pUC119 lysate, lysate of cells carrying pUC119 vector alone; pWD1 flscn-Seph, eluate of fluorescein-Sepharose incubation with French press lysate of cells carrying SCA-expressing pWD1 plasmid; pWD1 lysate, same as previous lane, prior to treatment with affinity matrix; pure 4-4-20; semi-

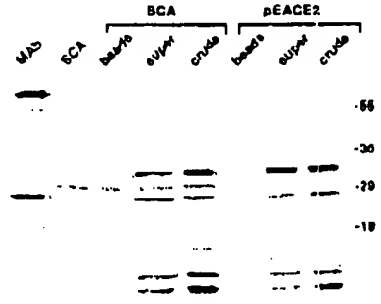

FIGURE 5 Immunoblot of 4-4-20 SCA gene products expressed in fission yeast. Signal visualization and probe as in Figure 4. Lanes contain (left to right): MAb, semi-purified 4-4-20 monoclonal antibody; SCA, 4-4-20 SCA synthesized in *E. coli*; SCA, protein products of fission yeast carrying SCA-encoding plasmid pBD1 from fluorescein Sepharose eluate (beads), supernatant from affinity matrix incubation (super) and cell lysate prior to incubation with matrix (crude); pEACE2 (beads, super, crude), same as previous three lanes except cells carried pEACE2 cloning vector lacking SCA gene insert.

here should greatly reduce the time required for construction of single chain antibody encoding genes.

EXPERIMENTAL PROTOCOL

Reagents and supplies. Oligonucleotides were synthesized by β-cyanoethylphosphoramidite chemistry on an Applied Biosystems Model 380A DNA synthesizer at the University of Illinois Biotechnology Center's Genetic Engineering Facility. Deprotected crude products were evaporated to dryness and precipitated three times in ethanol. In some instances, oligos were purified by reverse phase HPLC. Other reagent sources were as follows: cloned *Thermus aquaticus* (Taq) DNA polymerase, Perkin Elmer Cetus; modified T7 DNA polymerase ("Sequenase"), U.S. Biochemical; Novozyme, Calbiochem; Nusieve agarose, FMC; deoxynucleotides and random hexameric oligodeoxynucleotides, Pharmacia; RNAsin and immunological reagents, Promega; phenylmethylsulfonyl fluoride and leupeptin, Sigma. All other enzymes were obtained from Bethesda Research Laboratories. Nitrocellulose BA85 was obtained from Schleicher and Schuell.

Polymerase chain reaction. PCR syntheses were carried out in 100 μl reaction volumes in 0.5 ml microfuge tubes in either an MJR PTC-100 Thermal Cycler or a Perkin Elmer/Cetus Thermal Cycler. All PCR syntheses included 50 pmoles each of sense and anti-sense primers, approximately 1 nanogram of template, 250 μM of each dNTP, 1× PCR buffer consisting of 67mM Tris-HCl, pH 8.8, 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, and 10 mM β-mercaptoethanol and 2.5 U *Thermus aquaticus* DNA polymerase. Reaction mixtures were overlaid with 100 μl of mineral oil. All PCR profiles consisted of 1 min denaturation at 94°C, 2 min annealing at 60°C, and 1 min extension at 72°C. This sequence was repeated 25 times and was followed by a 5 min extension at 72°C. When PCR syntheses were performed with primary hybridoma cDNA products as templates, both reverse transcription of hybridoma RNA and PCR were performed sequentially in the same reaction tube[18]. 4-4-20 hybridoma RNA was purified as previously described[19]. One μg of total hybridoma mRNA was combined with 100 pmoles of random hexameric oligodeoxynucleotides, 200 U. Maloney Mul V reverse transcriptase, 20 U. RNAsin and 250 μM dNTPs in 1× PCR buffer in a final volume of 20 μl. The reaction mixture was incubated 1 hr at 42°C, heated for 5 min at 92°C and cooled to 4°C. To this mixture, primers and *T. aquaticus* DNA polymerase were added in 80 μl 1× Taq buffer and a PCR cycle profile was followed as described above. PCR primer sequences were as follows (5'-3'): VLSENSE1: GGGGATGCG TAACTAACTAAAGGAGAACAACAACAATGGATGTCGT-GATGACCCAAAC; VLSENSE2: CGGCATTGATCATATG-GATGTCGTGATGACCCAAAC; VLANTI: CTGAGT-AGATTTAGATTGAGAACCAGAACCAGAAGATTTACCT TCTTTGATCTCGAGCTTGGTGCCTCG; VHSENSE: GAAGGTAAATCTTCTTCGTTCTGGTTCTC-GAATCTAAATCTACTTCAGGAGGTGAAGCTTGAT-GAGACTGGA; VHANTI: GCGAATTCTTATGAGGAGACG-GTGACTGAGG.

Cloning of SCA-encoding genes in *E. coli*. The initial PCR-generated SCA-encoding structural gene was fused to the *lac Z* promoter of pUC119 for expression in *E. coli*. Products of PCR syntheses were immediately extracted with phenol:chloroform: isoamyl alcohol, then chloroform:isoamyl alcohol and were ethanol precipitated. PCR products and pUC119 were then digested with *BamHI* and *EcoRI*, enzymes whose recognition sequences had been incorporated into the distal PCR primers. Digestion products were gel-purified, combined and ligated in agarose[17]. Ligation products were transformed into *E. coli* XL1-Blue (Stratagene) using standard protocols[18]. Transformants were initially screened by restriction analysis of plasmids prepared by the rapid boiling method[19]. The verified product of this construction was named pWD1.

Cloning of SCA-encoding genes in *S. pombe*. The vector used for SCA gene expression in *S. pombe* was pEACE2. This vector was derived from pIRT5[20], a pUC118-derived translational fusion vector which carries a constitutive alcohol dehydrogenase (ADH) promoter and an *S. cerevisiae leu2* marker selectable by complementation of leucine auxotrophic fission yeast. pEACE2 includes these features plus an *S. pombe* autonomous replication sequence (ARS1) and an SV40 transcription termination sequence situated downstream from the ADH promoter and separated from the latter by an expanded multiple cloning site. The SCA-encoding gene in pWD1 was converted from a transcriptional to a translational fusion cassette by PCR. An oligonucleotide PCR primer (VLSENSE2) was prepared which, when combined in a PCR synthesis with the previously employed 3' (antisense) V_H primer (VHANTI) and pWD1, yielded a product suitable for ligation into pEACE2. VLSENSE 2 substitutes the 5' control sequences flanking the SCA cassette in pWD1 (see Results) with an *NdeI* restriction site, which contains in its recognition sequence (CATATG) the ATG start codon. This PCR product was purified and ligated into *NdeI/BglII*-digested pEACE2. The resulting construct, pBD1, was verified as above for pWD1. *S. pombe h-, leu1-32* was transformed with plasmid pBD1 by standard procedures[21] except that yeast were initially resuspended in 0.1 M lithium acetate, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and that 50% polyethylene glycol was added following mixing of plasmid and prepared yeast. Transformation mixtures were plated on agar containing 0.67% yeast nitrogen base without amino acids and 2% glucose. Putative transformants were verified by PCR as follows. A 5 ml culture of each isolate was grown to saturation in EMM-2 medium[22] and concentrated 5-fold by centrifugation and resuspension in cell wall digestion buffer (50 mM citrate-phosphate pH 5.6, 1.2 M sorbitol, 3 mg/ml Novozyme). Following 45 min incubation at 37°C, cells were washed and resuspended in 300 μl TE buffer with 200 mg of 0.1 mm glass beads. The suspension was agitated 30 sec in a Mini Bead-Beater (BioSpec) on high setting. The resulting lysate was incubated 5 min in boiling water and clarified by microcentrifugation at 13,000 × g for 5 minutes. One μl of clear supernatant was combined with PCR primers VLSENSE2 and VHANTI in PCR synthesis as described above. The products were visualized by conventional agarose gel electrophoresis and ethidium bromide staining. "No template" negative PCR controls were always included and yielded no product.

Expression of SCA-encoding gene in *E. coli* and yeast. Recombinant and control 4-4-20 SCA-encoding clones were grown at 37°C in LB broth to OD_{600} = 0.5-0.6 in *E. coli* XL1-Blue, whereupon IPTG was added to a final concentration of 0.4 mM to induce expression of the SCA gene under *lac* control. Following three hours of inductive growth, 2 ml of bacterial culture were concentrated 20-fold by centrifugation and resuspension in 1× protein sample buffer consisting of 62.5 mM Tris pH 6.8, 10% glycerol, 5% 2-mercaptoethanol and 2.3% sodium dodecyl sulfate. Thirty μl samples were electrophoresed in 15% SDS polyacrylamide gels[23] and separated proteins were electrophoresed in 15% SDS polyacrylamide gels[23] and separated proteins were electroblotted to nitrocellulose membranes overnight at 50V (constant voltage)[24]. Blots were probed with rabbit anti-idiotype polyclonal antiserum, directed against the murine IgG 4-4-20[10,22]. Positive antigen-antibody reactions were detected with secondary goat anti-rabbit IgG conjugated to alkaline phosphatase. Positive controls consisted of partially purified 4-4-20 IgG. Total fission yeast proteins were extracted in a manner similar to that described above for yeast nucleic acids. Cultures were grown to late exponential phase in YNB/AS medium (0.67% yeast nitrogen base without amino acids, 0.5% ammonium sulfate and 2.0% glucose) pelleted, resuspended at 100× concentration in extraction buffer consisting of 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.1% bromephenol blue and 10% glycerol. The cell suspension was then subjected to 1 min of agitation in the Mini Bead-Beater (high setting) and the lysate clarified by centrifugation.

Fluorescein Sepharose affinity chromatography. Fluorescein-Sepharose affinity matrix was a gift of Lisa Denzin. Cultures of *E. coli* carrying SCA gene constructs were grown in 250 ml volumes and induced as above. Cells were pelleted 5 min at 8000 × g and resuspended in 10 ml PBS (0.8% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.4) supplemented with 1.74 μg/ml phenylmethylsulfonyl fluoride and 8 μg/ml leupeptin. The suspension was passed through a French pressure cell at 1700 psi and the resultant lysate was clarified by centrifugation at 16,500 × g. The supernatant was saved and the pellet resuspended in 4 ml PBS. Both were stored at −20°C. Once the presence in the supernatant of SCA-immunoreactive material was confirmed (see Results), 4 ml of supernatant were combined with 0.5 ml packed PBS washed fluorescein Sepharose and incubated for 12-48 h in darkness at 4°C with gentle rocking. The matrix was washed twice in PBS and resuspended in 2× protein loading buffer. The suspension was then boiled, the matrix pelleted and a portion of the supernatant loaded onto an SDS polyacrylamide gel for electrophoresis and immunoblotting as described above. For affinity chromatographic analysis, total protein from lysates of *S. pombe* was prepared as above. The lysate was clarified by centrifugation and treated as described above for *E. coli* lysates.

DNA sequence analysis. All DNA sequencing was carried out on double stranded plasmid templates prepared by the rapid alkaline lysis method[25]. Sequencing protocols followed the modified T7 DNA polymerase enzyme supplier's recommendations.

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